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Application Number 10/596,692

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Hirabayashi et al.
Application No. 10/596,692
Filed: September 21, 2006
Confirmation No. 9539
For: METHODS FOR ANALYZING INTERACTIONS BETWEEN PROTEINS AND SUGAR CHAINS
Examiner: Bao-Thuy L. Nguyen
Art Unit: 1641
Attorney Reference No. 6235-85006-01

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DECLARATION OF DR. JUN HIRABAYASHI UNDER 37 C.F.R. § 1.132

1. I, Jun Hirabayashi, Ph.D., am a joint inventor of the above-identified patent application.

2. It is my understanding that the pending claims were rejected by the United States Patent and Trademark Office in the Office action dated September 15, 2010.

3. Experiments were carried out under my direction to determine the sensitivity of the claimed method for analyzing interaction between a sugar chain and a protein that interacts with a sugar chain.

4. The results of some of these experiments are submitted herewith as **Exhibits A** through **E**.

5. **Exhibit A** provides a schematic representation of an exemplary embodiment of the pending claims. Briefly, a glass slide with three lectins immobilized on the slide is contacted with a reaction solution containing a fluorescently labeled glyco-conjugate. Following incubation of the slide with the labeled glyco-conjugate, and *without* a washing step, an

excitation light is introduced from the edge of the glass slide, generating an evanescent wave close to the surface of the glass slide, and fluorescence is measured. The signal intensity correlates with the affinity of the glyco-conjugate for each lectin on the slide.

6. In one set of experiments, a lectin microarray was made as shown in **Exhibit B**. Different lectins were spotted on the microarray as shown in the diagram. The microarray also included non-lectin protein controls and Cy3-labeled bovine serum albumin (BSA) as a position marker. The microarray was probed with the listed fluorescently-labeled glycan probes. The microarray was contacted with two concentrations of each probe, 10 nM and 100 nM.

7. In one experiment, following incubation with the probes, a fluorescence image of the array was acquired using an evanescent-wave assisted scanner (GTMASScan III, Nippon Laser Electronics). An excitation light of 530 nm was introduced from the side edge of the slide. Scanning conditions of the intensified charge-coupled device camera (ICCD) were fixed: resolution, 5 μm; number of times for integration, 8; and exposure time, 110 msec. In a second experiment, after the incubation, each well was washed 3 times with TBS containing 0.05% Tween® 20. A fluorescence image of the microarray was immediately acquired using a conventional confocal-type scanner (GenePix 4000B, Axon).

8. As shown in **Exhibit C**, utilizing the evanescent wave method (middle panel), sensitive and specific detection of glycan binding to the lectins on the microarray was achieved with the 10 nM probe concentration (upper portion). The net signal intensity was 9604 for detection of ConA with TAMRA-labeled M6. Using the 100 nM probe concentration produced fluorescence that was above the upper-limit of detection for the scanner (lower portion). In contrast, utilizing the conventional confocal method (right panel) no detectable signal from the glycan probes was observed (upper portion). Weak signal was detectable for ConA/TAMRA-M6 (net intensity 1500) when the 100 nM probe concentration was utilized (lower portion). Thus, the current method could achieve at least about 6-fold higher signal with 10-fold less probe than the conventional confocal method.

9. In further experiments, lectin microarray was made by immobilizing RCA120 lectin on a glass slide coated with 3-glycidoxypropyl trimethoxysilane. Individual wells were formed on the slide by affixing a black silicone rubber having a number of openings on the glass slide. Tetramethylrhodamine-labeled NA2 glycan (TMR-Asn-NA2) dissolved in Tris-buffered saline (TBS) containing 0.05% Tween® 20 was applied to each well of the slide (100 µl of 100 nM solution). The slide was then incubated at room temperature until the binding reaction reached equilibrium (200 minutes). A fluorescence image of the array was acquired using an evanescent-wave assisted scanner. An excitation light of 530 nm was introduced from the side edge of the slide. Scanning conditions of the intensified charge-coupled device camera (ICCD) were fixed: resolution, 5 µm; number of times for integration, 8; and exposure time, 110 msec.

10. A lectin microarray as described in paragraph 9 was also used for a comparison of the method with standard methods utilized in the field. TMR-Asn-NA2 dissolved in TBS containing 0.05% Tween® 20 was applied to each well of the slide (100 µl of 100 nM solution). The slide was then incubated at room temperature until the binding reaction reached equilibrium (200 minutes). After the incubation, each well was washed 3 times with TBS containing 0.05% Tween® 20. A fluorescence image of the microarray was immediately acquired using an evanescent-wave assisted scanner. As described in paragraph 6, an excitation light of 530 nm was introduced from the side edge of the slide. Scanning conditions of the intensified charge-coupled device camera (ICCD) were fixed: resolution, 5 µm; number of times for integration, 8; and exposure time, 110 msec.

11. As shown in **Exhibit D**, when the method included the washing step, only weak fluorescence was detectable (right panel). However, the omission of the washing step resulted in robust fluorescence. This confirmed the increased sensitivity of the method lacking a washing step.

12. In another experiment, a microarray as described in paragraph 9 was contacted with 100 µl of Cy3-labeled ASF glycoprotein (100 ng/ml) in TBS containing 0.05% Tween® 20. The array was incubated at room temperature until the binding reaction reached equilibrium. After 125 minutes, the Cy3-ASF containing solution was displaced with TBS containing 0.05%

Tween® 20. A fluorescence image of the microarray was continuously acquired every minute using an evanescent-wave assisted scanner. An excitation light of 530 nm was introduced from the side edge of the slide. Scanning conditions of the intensified charge-coupled device camera (ICCD) were fixed: resolution, 5 μm; number of times for integration, 8; and exposure time, 110 msec. All data were imported in TIFF format and analyzed with Array-Pro® Analyzer version 4.5 (Media Cybernetics, Inc., Silver Spring, MD). The net intensity value for each spot was calculated on the basis of the signal intensity minus the background value. Five replicates of net signal intensity values were averaged.

13. As shown in **Exhibit E**, the signal intensity increased in a time-dependent manner, and eventually became saturated within 120 minutes. After replacement of the Cy3-ASF solution with wash buffer, the signal intensity decreased in a time dependent manner. Part A shows a scanning image at various time points after addition of Cy3-ASF. Part B shows the time course of the RCA120-ASF interaction. The error bars represent the standard deviation of five replicate spots.

14. The experiments described above demonstrate that an interaction between a sugar chain and a protein that interacts with a sugar chain can be determined with a high level of sensitivity and also that such interactions can be measured in real-time in a quantitative manner.

15. In the experiments described above, reaction vessels were formed on the glass slide using a rubber sheet with multiple holes by affixing the rubber sheet to the glass slide. This provides for a high-throughput system for analyzing the weak interactions between sugar chains and proteins that interact with sugar chains. Conventional multi-well plastic plates are not suitable for the methods disclosed in the application, because they are bulky and non-flat, and produce both stray light and non-homogenous evanescent waves, which result in high background. However, utilizing a rubber sheet and a glass substrate provides a flat surface (the glass substrate) and an exact fit to the substrate of the rubber sheet. This does not interfere with the generation of the evanescent wave by injection of the excitation light from the edge of the glass substrate.

16. In summary, the claimed methods and substrates provide easier, faster, more sensitive, and more accurate methods for analyzing interactions between sugar chains and proteins that interact with sugar chains than conventional methods. In particular, this is made possible by use of the glass substrate and rubber sheet and by measuring the intensity of excited fluorescence after applying an evanescent wave generated by injecting an excitation light from the edge of the glass substrate.

17. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Jun Hirabayashi, Ph.D.

14th, Feb. 2011
Date